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Immunology

Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin

(cyclosporin A/FK506 binding protein/rotamase)

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ABSTRACT **Proliferation and immunologic function of T lymphocytes are initiated by signals from the antigen receptor that are inhibited by the immunosuppressant FK506 but not by its structural analog, rapamycin. On the other hand, interleukin 2 (IL-2)-induced signals are blocked by rapamycin but not by FK506. Remarkably, these two drugs inhibit each other's actions, raising the possibility that both act by means of a common immunophilin (immunosuppressant binding protein). We find that the dissociation constant of rapamycin to the FK506 binding protein FKBP (K_d = 0.2 nM) is close to the dissociation constant of FK506 to FKBP (K_d = 0.4 nM) and to their effective biologic inhibitory concentrations. However, an excess of rapamycin is needed to revert FK506-mediated inhibition of IL-2 production, apoptosis, and transcriptional activation of NF-AT, a T-cell-specific transcription factor necessary for IL-2 gene activation. Similarly, an excess of FK506 is needed to revert rapamycin-mediated inhibition of IL-2-induced proliferation. The drug concentrations required for antagonism may be explained by the relative affinity of the drugs to, and by the abundance of, the immunophilin FKBP. FKBP has been shown to catalyze the interconversion of the cis- and trans-rotamers of the peptidyl-prolyl amide bond of peptide substrates; here we show that rapamycin, like FK506, is a potent inhibitor of the rotamase activity of FKBP (K_i = 0.2 nM). Neither FKBP binding nor inhibition of rotamase activity of FKBP alone is sufficient to explain the biologic actions of these drugs. Rather, these findings suggest that immunophilin bound to FK506 interferes with antigen receptor-induced signals, while rapamycin bound to the immunophilin interferes with IL-2-induced signals.**

Cyclosporin A (CsA) and the structurally unrelated immunosuppressant FK506 have been demonstrated to inhibit T-cell activation and to be effective in organ transplantation (1–3). CsA has been shown to bind to and inhibit the rotamase activity of a cytoplasmic enzyme, cyclophilin (CyP) (4–6). Recently, a cytoplasmic receptor for FK506, FK506 binding protein (FKBP), which is unrelated in sequence to CyP, has also been shown to have similar rotamase activity, suggesting that this common enzymatic property of immunophilin proteins (immunosuppressant binding proteins) may be related to the regulation of T-cell activation (7–10). Rapamycin is a macrolide similar in structure to FK506 and, like FK506, inhibits T-cell proliferation (11, 12) and binds to FKBP.

Recently, rapamycin and FK506 have been shown to inhibit each other's actions (12, 13). Here we confirm and extend those findings by examining a number of specific actions of these drugs. Our results suggest that a common drug receptor binding site is involved in at least two distinct signaling pathways in the T-cell activation cascade. Both rapamycin (K_d = 0.2 nM) and FK506 (K_d = 0.4 nM) are shown to bind with high affinity to FKBP. Thus, FKBP is possibly involved in one or both of the aforementioned pathways. In addition, both rapamycin (K_i = 0.2 nM) and FK506 (K_i = 1.7 nM; ref. 14) are shown to be inhibitors of the rotamase activity of FKBP. Accordingly, the inhibition of the rotamase activity of FKBP, assayed *in vitro* with synthetic substrates, is apparently an insufficient requirement for mediating the biological effects of either rapamycin or FK506.

MATERIALS AND METHODS

Reagents, Cell Culture, Interleukin 2 (IL-2) Production, and DNA Fragmentation. The IL-2-producing murine T-cell hybridoma 16.CD2-15.20 (15) was cultured (10^6 cells per well) with or without a 2% (vol/vol) culture supernatant of the anti-murine CD3 monoclonal antibody (mAb) 145-2C11 (16) in the absence or presence of CsA, FK506, or rapamycin. At 24 hr, culture supernatants were dialyzed and assayed for the presence of IL-2 as described (15). DNA was isolated from the cells and assayed for induction of DNA fragmentation or apoptosis on an ethidium bromide-stained 2% agarose gel as described by Shi *et al.* (17).

NFAT-Gal Plasmid and Transfection into Jurkat Cells. The NFAT-Gal plasmid and Jurkat cell line stably transfected with the plasmid were described by Fiering *et al.* (18). The plasmid contains the *Escherichia coli lacZ* gene attached to a minimal promoter of the IL-2 gene (−72 to +47), linked to a trimer of the NF-AT (a T-cell-specific transcription factor) binding site (−286 to −257 of IL-2), and a hygromycin-resistance gene linked to thymidine kinase enhancer (see Fig. 2*A*). Twenty micrograms of linearized plasmid was electroporated into Jurkat cells (250 V and 960 μF of electric shock in 1 ml of RPMI 1640), and stable transfectants were established after hygromycin selection. Clones that expressed the highest β-galactosidase activity upon stimulation were selected using the fluorescence-activated cell sorter and the fluorogenic substrate fluorescein di-β-D-galactoside (18, 19). Ribonuclease protection assays were performed by using the template described by Verweij *et al.* (20) as described by Melton *et al.* (21).

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Abbreviations: CsA, cyclosporin A; CyP, cyclophilin; FKBP, FK506 binding protein; IL-2, interleukin 2; mAb, monoclonal antibody.

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EXHIBIT C

Measurement of β-Galactosidase Activity, Nuclear Extracts, and DNA Binding Studies. β-Galactosidase activity was measured from the fluorescence of 4-methylumbelliferyl β-D-galactoside (Sigma) cleaved at the β-galactosidase bond. Fifty thousand cells were stimulated in 200 μl of fresh culture medium and centrifuged; the supernatant was removed; and 180 μl of reaction medium (100 mM Na_2HPO_4, pH 7.0/10 mM KCl/1 mM $MgSO_4$/0.1% Triton X-100/0.5 mM 4-methylumbelliferyl β-D-galactoside) was added. After 1 hr at 20°C the reaction was stopped by adding 75 μl of stop buffer (300 mM glycine/15 mM EDTA, pH 11.3), and fluorescence at 460 nm was measured by using 355-nm excitation with a Titertek Fluoroscan II. Nuclear extracts were made as described (22). Protein samples were desalted by passage over a Bio-Gel P-6DG column (Bio-Rad) at 4°C. Protein concentrations were determined by the Bradford assay (23). Electrophoretic mobility assays were performed as described (24, 25) with the sequences shown in Fig. 2 as probe for NF-AT and 10 μg of protein.

Competitive Binding to FKBP. Assays by the Sephadex LH-20 method (4) measured the displacement of [^{3}H]dihydroFK506 from recombinant human FKBP (9) by dihydroFK506, rapamycin, and FK506. Protein (1 nM), labeled drug (0.5 nM), and competitor were incubated for 30 min in 110 μl of buffer (100 mM NaCl/20 mM phosphate/1 mM EDTA, pH 7.3) to which 0.015% Triton X-100 was added. The bound fraction was isolated by chromatography of 50 μl of the mixture on a 1.5-ml column by Sephadex LH-20 (Pharmacia). This sample and 50 μl of assay mixture were assayed by scintillation spectrometry to obtain the bound and total concentrations, respectively. Binding data were analyzed with the LIGAND-PC program (26).

Rotamase Activity and Inhibition Studies. The assay was performed as described (6) with the test peptide succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide (14, 27) (Bachem) and recombinant human FKBP. A 3.5-ml cuvette was charged with 1.4 ml of test peptide (55 μM) in 40 mM Hepes (pH 7.9) plus 0.015% Triton X-100. Stirring was begun and FKBP (5.0 μl of a 10.8 μM solution) and rapamycin solution [in 25 μl of 50% (vol/vol) methanol] were added. α-Chymotrypsin was added (75 μl of 10 mg/ml in 1 mM HCl) and the A_{410} (*p*-nitroaniline) was monitored; data were collected every 0.5 s. The temperature was maintained at 10.0 ± 0.1°C. First-order rate constants were calculated by using the KORE program (28). The data were fit to a tight-binding inhibitor model (29), from which the K_i of 0.2 nM and the FKBP concentration of 36 nM were derived.

RESULTS

We have used a murine T-cell hybridoma as a model of antigen-specific T-cell activation. The murine T-cell hybridoma 16.CD2-15.20 can be activated to produce IL-2 by means of the cell surface T-cell receptor–CD3 complex either by antigen or by mAb directed against CD3 (anti-CD3) (15). Also, upon stimulation there is a diminution of the proliferative rate as well as nuclear lobulation, DNA fragmentation, and cell death by apoptosis (17, 30).

We have assessed the effect of FK506 and rapamycin on the functional properties of this T-cell hybridoma (Fig. 1). The ability of the murine T-cell hybridoma to produce IL-2 upon stimulation with anti-CD3 was inhibited by FK506 (Fig. 1*A*) in a concentration-dependent fashion (data not shown). As has been observed in other systems (31, 32), FK506 was 10- to 100-fold more effective than CsA in inhibiting IL-2 production. Rapamycin, structurally related to FK506, has been shown to act as an immunosuppressant and to inhibit T-cell proliferation (11–13). However, anti-CD3-induced IL-2 production by the hybridoma was not significantly inhibited by rapamycin (Fig. 1*A*).

Incubation with anti-CD3 decreased the rate of proliferation of the hybridoma (data not shown) and induced apoptosis or DNA fragmentation to characteristic multimers of 180 base pairs (Fig. 1*B*). The addition of 1 nM FK506 at the initiation of culture completely prevented the induction of apoptosis. However, rapamycin, even at 100-fold higher concentrations, did not prevent IL-2 production or activation-induced cell death.

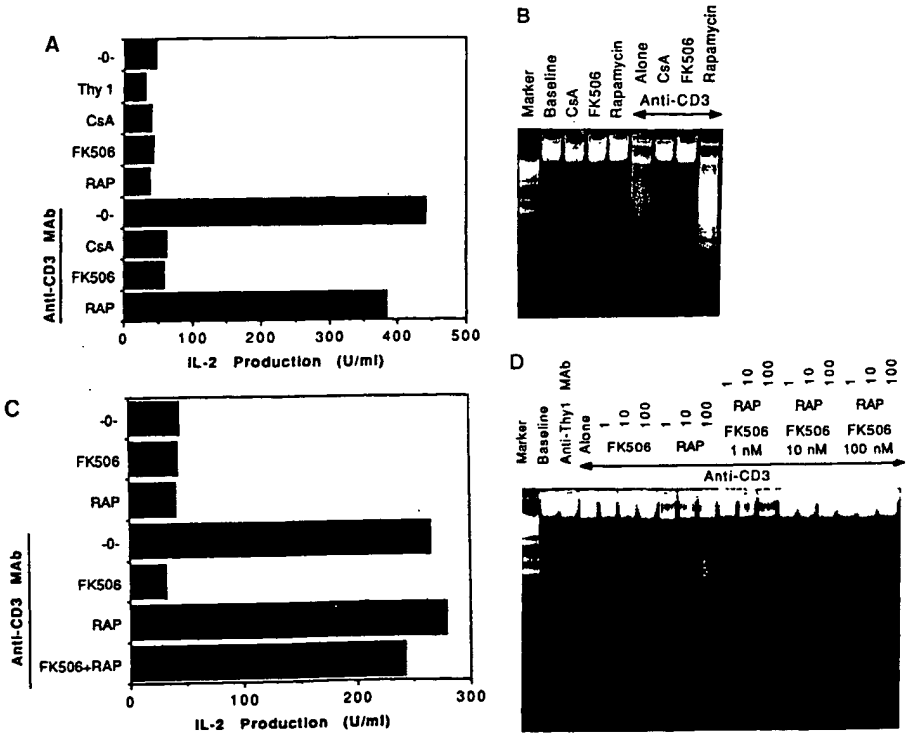

FIG. 1. Effect of FK506 and rapamycin on IL-2 production (*A* and *C*) and activation-induced cell death (*B* and *D*). The murine T-cell hybridoma 16.CD2-15.20 (15) was cultured with the anti-murine CD3 mAb 145-2C11 (16), in the absence or presence of 10 nM CsA, 1 nM FK506, or 100 nM rapamycin (RAP) except as indicated. At 24 hr, dialyzed culture supernatants were harvested and assessed for the presence of IL-2 by their ability to support the proliferation of an IL-2-dependent murine T-cell line, CTLL-20 (*A* and *C*); remaining cells were assessed for induction of DNA fragmentation or apoptosis on an ethidium bromide-stained 2% agarose gel (*B* and *D*). Anti-Thy 1 and anti-CD3 mAb were used at 2% (vol/vol) culture supernatant.

The addition of rapamycin at a 100-fold excess concentration effectively inhibited the ability of FK506 to prevent activation-induced cell death (Fig. 1*D*). Furthermore, rapamycin effectively reversed FK506-mediated inhibition of IL-2 production by the hybridoma (Fig. 1*C*). Thus, rapamycin itself did not affect T-cell receptor–CD3-mediated signal transduction but did reverse the effect of FK506 upon this pathway.

Recent evidence indicates that the IL-2 gene is under the control of a T-cell-specific transcription factor (NF-AT) that is activated after antigen receptor stimulation of T cells (33). Although other transcription factors bind to the IL-2 enhancer (34–36), they are expressed in many cell types or are activated without a requirement for protein synthesis. Since IL-2 gene activation requires protein synthesis and occurs only in T lymphocytes, NF-AT is likely to be one protein that gives rise to the restricted expression of IL-2. We investigated the effects of FK506 and rapamycin on NF-AT-dependent transcription by assaying β-galactosidase mRNA expression and enzymatic activity in a human T-cell leukemia Jurkat cell clone stably transfected with a construct in which a trimer of the 30-base-pair NF-AT binding site directs transcription of the β-galactosidase gene (Fig. 2*A*). FK506 completely inhibited β-galactosidase mRNA expression at 1.2 nM, and rapamycin (550 nM) was able to completely reverse the effects of 1.2 nM FK506 (Fig. 2*B*). Stimulation of cells in the presence of rapamycin with various concentrations of FK506 provided a dose-dependent shift of the FK506 dose–response curve in which a 10-fold change in the concentration of rapamycin produced a 10-fold reduction in the sensitivity to FK506 (Fig. 2*C*). At the 50% inhibition level, ≈300-fold more rapamycin, compared to FK506, was required to reverse the effect of FK506 (Fig. 2*C*). Rapamycin alone had no effect on β-galactosidase expression at concentrations up to 1.1 μM. FK506 (1.2 nM) reduced the induction of NF-AT binding activity by about 10-fold (Fig. 2*D*), which is substantially less than the 1000-fold or more reduction in transcriptional activity of this protein (Fig. 2*B*). Rapamycin was able to overcome completely the reduction in NF-AT binding activity produced by FK506 (Fig. 2*D*). Thus, the inhibitory effect of FK506 is more marked on IL-2 transcription than on NF-AT binding.

CsA has been shown to block completely the transcriptional activity of NF-AT and to block partially the development of DNA binding activity (37). Unlike its effects on FK506, rapamycin was not able to reverse the effects of CsA on NF-AT-dependent transcription or the reduction of NF-AT binding activity seen with CsA (data not shown).

Rapamycin, but not FK506, inhibited IL-2-driven proliferation of a cloned, IL-2-dependent T-cell line, CTLL-20 (Fig. 3), suggesting that, compared to FK506, rapamycin has a different inhibitory effect on T cells. The addition of FK506 at 100-fold excess concentrations to rapamycin-treated cultures of CTLL-20 restored T-cell proliferation (Fig. 3), supporting a distinct, and separable, spectrum of action for rapamycin and FK506 and suggesting that the basis of immunosuppression by FK506 and rapamycin differ.

The mutual inhibition of FK506 and rapamycin suggests the existence of a common receptor binding site. Recently,

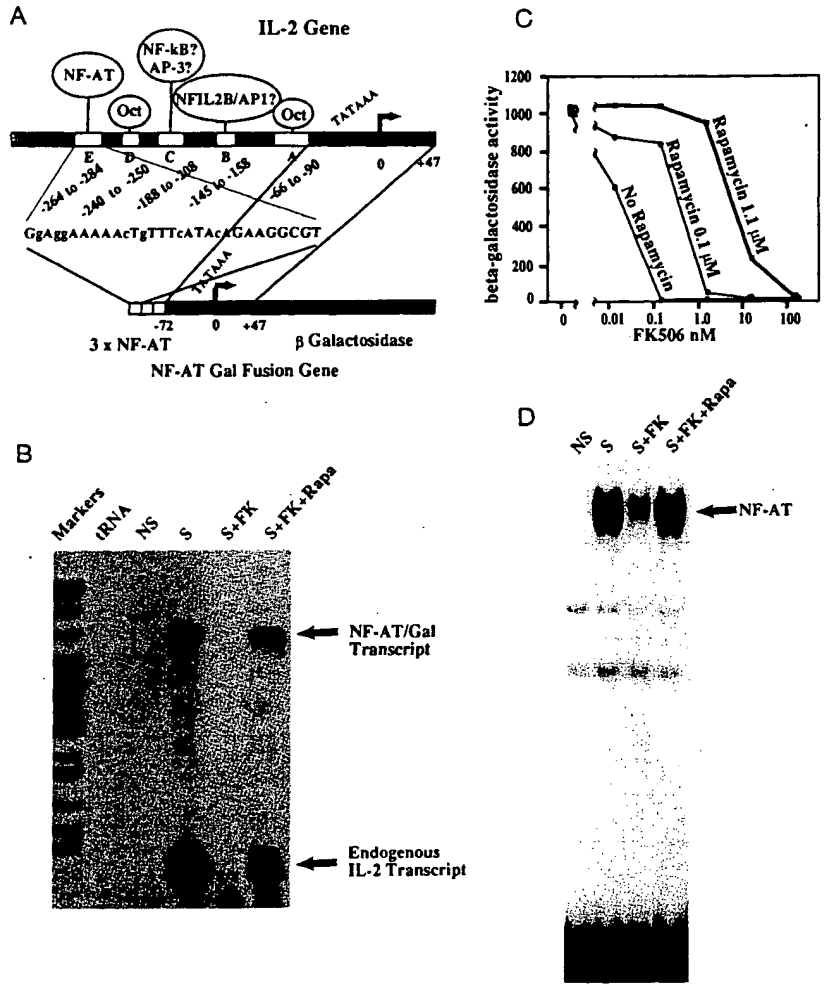

FIG. 2. (*A*) Production of the NFAT-Gal construct. The putative binding protein sites on the IL-2 gene are noted. The NFAT-Gal construct contains *E. coli lacZ* attached to the IL-2 gene at position +47. The promoter is the fragment of IL-2 from −72 to +47, which contains a TATA box. A trimer of the NF-AT binding site (−286 to −257 of IL-2), oriented 5′ to 3′, is ligated to the IL-2 promoter at −72. Lowercase letters in the NF-AT sequences are bases where methylation inhibits DNA binding. (*B*) FK506 inhibits transcriptional activity of NF-AT and transcription of IL-2 mRNA, whereas rapamycin reverses the effects of FK506. Transcription of β-galactosidase mRNA from the NFAT-Gal construct was measured by a ribonuclease assay. Transcripts from the endogenous IL-2 gene are measured as a 47-base-pair transcript, whereas properly initiated transcripts from the transfected gene are 250 base pairs. NS, nonstimulated cells; S, cells stimulated for 5 hr with 32 nM phorbol 12-myristate 13-acetate and 2 μM ionomycin; FK, 1.2 nM FK506; Rapa, 550 nM rapamycin; Markers, *Msp* I-digested pBR322 DNA. (*C*) Dose–response curve of effects of FK506 and rapamycin on the transcriptional activity of NF-AT, measured as the enzymatic activity of β-galactosidase transcribed from the NFAT-Gal construct. Jurkat cells were stimulated as in *B*. (*D*) FK506 partially inhibits the development of DNA binding activity of NF-AT, and rapamycin reverses the effect of FK506. Jurkat cells, 5×10^7 for each condition, were stimulated for 5 hr with 32 nM phorbol 12-myristate 13-acetate and 2 μM ionomycin. NF-AT binding activity was assayed by electrophoretic mobility shift assay using the NF-AT binding site as a probe (20). Abbreviations are as in *B*.

the predominant cytosolic binding protein for FK506, FKBP, was isolated and shown to be a rotamase (7–9). FK506, but not CsA, binds FKBP with high affinity and potently inhibits this rotamase activity. Recently we have observed that FKBP was also the predominant protein isolated with a rapamycin affinity column (H. Fretz, M. W. Albers, R.F.S., A. Galat, and S.L.S., unpublished results). In a competitive binding assay, rapamycin (K_d = 0.2 nM) was slightly more effective than FK506 (K_d = 0.4 nM) in displacing [^{3}H]dihydroFK506 from recombinant human FKBP (Fig. 4). Both rapamycin (K_i = 0.2 nM) and FK506 (K_i = 1.7 nM; ref. 14) are also potent rotamase inhibitors of FKBP (Fig. 5).

DISCUSSION

We have confirmed that FK506 and rapamycin act upon distinct T-cell signal transduction pathways and inhibit each other's actions. FK506 inhibits IL-2 production and activation-induced apoptosis in a constitutively dividing T-cell line; rapamycin reverses the actions of FK506. In addition, rapamycin restores NF-AT-dependent transcriptional activation and DNA binding inhibited by FK506. Although rapamycin-induced inhibition of lymphokine-dependent proliferation was only modest (30%, Fig. 3), the addition of FK506 consistently reversed this inhibition. These data are consistent with prior studies demonstrating that FK506 reversed the suppressive effect of rapamycin (40–60%) on the stimulation of splenic T cells with IL-2 plus phorbol 12-myristate 13-acetate (13). The observation that rapamycin does not inhibit NF-AT-directed transcriptional activation (Fig. 2) further supports the conclusion that FK506 and rapamycin affect different signal transduction pathways. Both FK506 and rapamycin bind to and inhibit the rotamase activity of the immunophilin FKBP. A common binding site for both drugs on FKBP has been determined by NMR spectroscopic studies of the rapamycin–FKBP and FK506–FKBP complexes (S. W. Michnick, M. K. Rosen, T. J. Wandless, and S.L.S., unpublished results).

The concentration required for reciprocal inhibition between FK506 and rapamycin in biological assays suggests that the receptor site, and not the drug, is in excess in the cell. This is readily explained by the abundance of the immunophilin FKBP (≈5 nM; ref. 38), which acts as a buffer against the mutually inhibitory actions of the two drugs. The concentration of the antagonizing agent rises to sufficient levels to displace the drug effectively from its biological receptor only after the excess FKBP binding sites are occupied. This

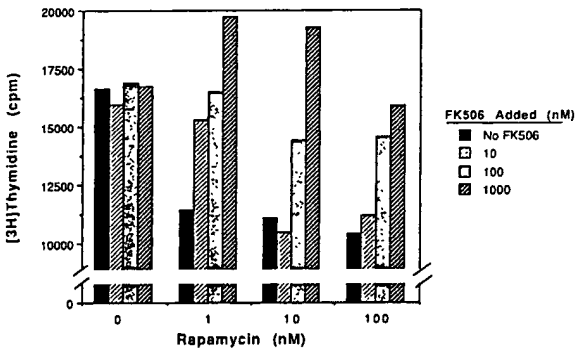

FIG. 3. FK506 reverses the inhibition mediated by rapamycin. Rapamycin inhibition of, and FK506 reversal of, IL-2-dependent proliferation of CTLL-20 is shown. The murine IL-2-dependent T-cell line CTLL-20 was cultured in the presence of 0.5% IL-2-containing rat culture supernatant in the absence or presence of rapamycin or FK506 at the indicated concentrations. Proliferation was assessed by the incorporation of [^{3}H]thymidine.

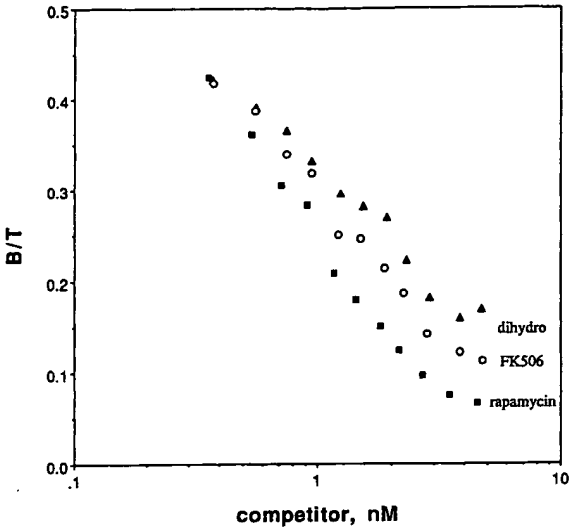

FIG. 4. Competitive binding to FKBP. The binding assay measured the ability of FK506 (K_d = 0.4 nM), rapamycin (K_d = 0.2 nM), and dihydroFK506 (K_d = 0.8 nM) to displace [^{3}H]dihydroFK506 from FKBP, and the fraction of total label bound is shown versus total competitor concentration. Each point is the average of two measurements. B, bound; T, bound plus free, or total.

concentration dependence and the above analysis also strongly suggest that the immunophilin–drug complex is the biological effector. This conclusion is analogous to that reached by Tropschug *et al.* (39) in their study of mutant strains of *Neurospora crassa* and *Saccharomyces cerevisiae* that are resistant to CsA. These mutated organisms either lost their ability to produce the CsA binding immunophilin CyP, or, if CyP was produced, it no longer bound CsA. Thus, it was concluded that the immunophilin–drug complex was responsible for the toxic actions of the drug on these organisms. Invoking the immunophilin–drug complex as the biological effector addresses the issue of how the ubiquitous CyP and FKBP immunophilins could be involved in the T-cell activa-

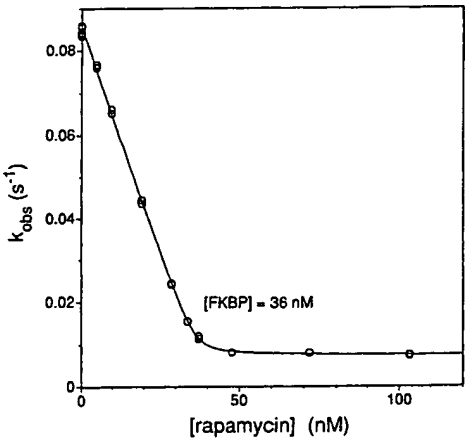

FIG. 5. Inhibition of rotamase activity of FKBP by rapamycin. The first-order rate constant for cis → trans isomerization of the test substrate in the presence of 36 nM FKBP is plotted against drug concentration. Two points are shown for each drug concentration, and five points are shown for the control (no drug). The curve is the best-fit binding model. The horizontal asymptote represents the rate of isomerization in the absence of catalysis (k = 0.0074 s^{-1}). obs, Observed.

tion process. It is possible that these proteins have a more general cellular function, perhaps assisting in protein folding *in vivo* (as foldases). Only when the immunophilin combines with its immunosuppressive ligand does it inhibit T-cell activation. We suggest that the cellular immunophilin receptor, bound to either rapamycin or FK506, may interact with different molecules in separable pathways of T-cell activation.

The above hypothesis raises the question as to whether the immunosuppressive drugs may mimic the actions of endogenous immunophilin ligands. If so, the active site of a rotamase enzyme would also serve as a regulatory binding site for use in immune modulation. Rotamase inhibition should not necessarily correlate with immunosuppression, as effective drug concentrations of 0.5 nM would only inhibit ≈10% of the total enzyme present in the cell. Indeed, both rapamycin and FK506 inhibit the rotamase activity of FKBP but have different effects on T-cell activation. These results suggest that inhibition of the rotamase activity of FKBP assessed *in vitro* cannot explain the mechanism of action of these compounds, as we have recently shown (40).

FKBP is the predominant protein isolated from both FK506 affinity and rapamycin affinity matrices. From both matrices, however, other, low-abundance FK506 and rapamycin binding proteins have been eluted and characterized (H. Fretz, M. W. Albers, R.F.S., A. Galat, and S.L.S., unpublished results). Thus, the possibility also exists that the common binding site implied by the mechanistic studies reported herein and elsewhere (13) is found on separate immunophilin proteins. In this circumstance, the high affinity of FK506 and rapamycin for FKBP and the high abundance of FKBP require that an even higher affinity for drug must exist with the minor immunophilins or that the intrinsic activity of the drugs is actually much greater than the apparent activity measured experimentally. In either situation, the low-abundance immunophilin must compete with FKBP for drug and thus overcome the buffer effect seen in the mutual inhibition studies.

These data support several conclusions. Neither mere binding to FKBP nor rotamase inhibition can explain the effects of FK506 and rapamycin on T-cell activation. The high affinity of FKBP for both agents makes it an attractive, but not unique, candidate for the relevant *in vivo* receptor of both drugs. This apparently common receptor site must be involved in regulating at least two different signal transduction pathways, one initiated at the T-cell receptor and resulting in lymphokine production and the other, perhaps initiated at a lymphokine receptor and resulting in cellular proliferation. A comparison of the FK506 and rapamycin structures suggests that they consist of a common receptor-binding domain (recently suggested to mimic a twisted leucyl-prolyl amide bond) (27) and unique effector domains that are responsible for the difference in their activities. The effects of the two drugs would then arise from interactions of their respective receptor complexes with different target molecules.

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affinity purification procedure using lysates from [^{35}S] methionine-labelled cells (data not shown). The GFK–ketorapamycin and GFK–isorapamycin complexes bound FRAP less effectively than GFK–rapamycin; at concentrations of 500 nM, the keto- and iso- complexes were unable to retain the 220K protein (Fig. 1*a*, lanes 4, 5), whereas at higher concentrations of the complexes (2.5 μM) detectable quantities of FRAP were retained (Fig. 1*a*, lanes 4, 5). This is consistent with the finding that these compounds are still strong cell-cycle inhibitors, albeit less potent than rapamycin itself. Thus, the binding of GFK–ligand complexes to FRAP correlates with the ability of the ligands to impede G1 progression in MG-63 cells. FRAP was also detected in Jurkat *T*-lymphocyte cells and rat basophilic leukaemia cells (Fig. 1*b*), two mammalian cell lines that are also sensitive to rapamycin[6,22]. No other rapamycin-specific bands were observed in each case.

FRAP purified from bovine brain (bFRAP) had a similar specificity for GFK-ligand (Fig. 2*a*). Microsequencing of bFRAP proteolytic fragments (298 amino acids in total, Fig. 2*b*) led to the design of a pair of degenerate oligonucleotides for use in the polymerase chain reaction (PCR). A 182 bp PCR product allowed for the isolation of overlapping clones from a human Jurkat T cell λZAP II cDNA library, yielding 7.6 kb of contiguous sequence. Using these cDNA sequences as probes, a band migrating at approximately 8.5 kilobases was detected by Northern blot analysis of oligo dT purified RNA isolated from a variety of human tissues and cell lines (Fig. 2*c*). The human cDNA sequence encodes an amino-acid open reading frame (ORF) and aligns with 99% identity to the bFRAP peptides (Fig. 2*b*). As N-terminal peptide sequence from purified bovine FRAP was not obtained, the initiating methionine shown in Fig. 2*b* is unconfirmed. The predicted molecular mass of this ORF (~300K) is greater than that inferred by the mobility of FRAP during SDS–PAGE (above).

Human FRAP is highly related to the *DRR1/TOR1* and *DRR2/TOR2* gene products. Overall it is 44% identical to DRR1/TOR1 and 46% identical to DRR2/TOR2. The region of greatest homology to DRR1/TOR1 and DRR2/TOR2 lies in the C-terminal 660 amino acids of human FRAP (57% and 59% identical, respectively). In addition, this region has homology to several known phosphatidylinositol kinases (21% identity on average), including mammalian phosphatidylinositol 3-kinase[17,18] (PI3K), a yeast PI3K *VPS34* (refs. 17 and 18) and *PIK1* (ref. 20). These similarities indicate that FRAP may also have phosphatidylinositol kinase activity.

Through the introduction of minute structural changes in rapamycin, this study implicates FRAP as a mediator of G1 cell cycle progression in mammalian cells. Identification of FRAP as the target of FKBP12-rapamycin together with the earlier demonstration of calcineurin as the target of FKBP12-FK506 (ref. 2) addresses a fascinating aspect of immunophilin research, namely that the immunophilin FKBP12 can bind two distinct natural products and thereby gain the ability to bind two distinct signalling molecules involved in cell cycle entry and progression. Further biochemical characterization of this unique mammalian protein should elucidate its role in propagating the mitogen-initiated signals that lead to the activation of p70^{S6k} and cyclin–Cdk complexes. □

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Functional dissection of the yeast Cyc8–Tup1 transcriptional co-repressor complex

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DNA-BINDING repressor proteins mediate regulation of yeast genes by cell type (Mcm1/α2 and a1/α2), glucose (Mig1) and oxygen (Rox1) (refs 1–4 respectively). An unusual feature of all these regulatory pathways is that transcriptional repression requires two physically associated proteins[5] that do not bind DNA Cyc8(Ssn6) and Tup1. The Cyc8–Tup1 complex has been proposed to be a co-repressor that is recruited to target promoters by pathway-specific DNA-binding proteins[6], but the specific functions of the individual proteins are unknown. Here we show that when it is bound upstream of a functional promoter through the LexA DNA-binding domain, Tup1 represses transcription in the absence of Cyc8. Deletion analysis indicates that Tup1 contains at least two non-overlapping transcriptional repression regions with minimal primary sequence similarity, and a separable Cyc8-interaction domain. These Tup1 domains, which do not include the β-transducin motifs[7], are necessary and partially sufficient for Tup1 function. We suggest that Tup1 performs the repression function of the Cyc8–Tup1 co-repressor complex, and that Cyc8 serves as a link with the pathway-specific DNA-binding proteins.

It has been previously shown that Cyc8 can repress transcription in a Tup1-dependent manner when bound upstream of the intact *CYC1* promoter through the heterologous LexA DNA-binding domain[6]. Similarly, a LexA–Tup1 hybrid protein confers a 16-fold reduction of expression from a promoter containing four LexA operators upstream of the *CYC1* promoter (Table 1). LexA–Tup1 and LexA–Cyc8 also repress expression of a *his3* gene containing a single LexA operator upstream of the T_R TATA element (Fig. 1*a*), suggesting that they can inhibit basal transcription. Surprisingly, LexA–Tup1 retains almost its entire

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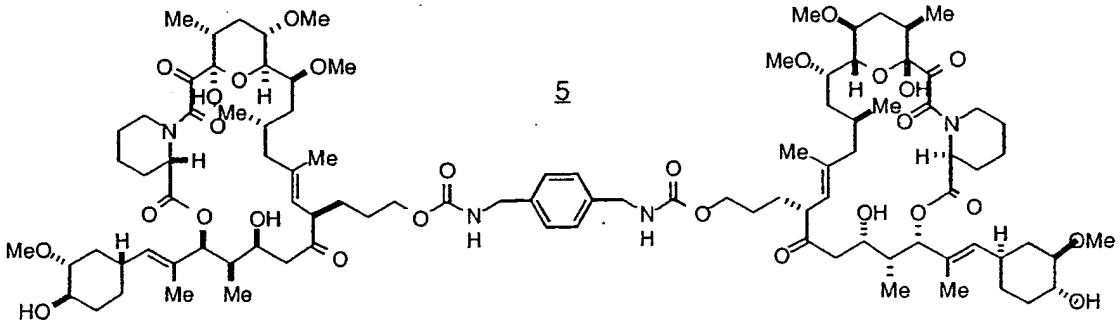

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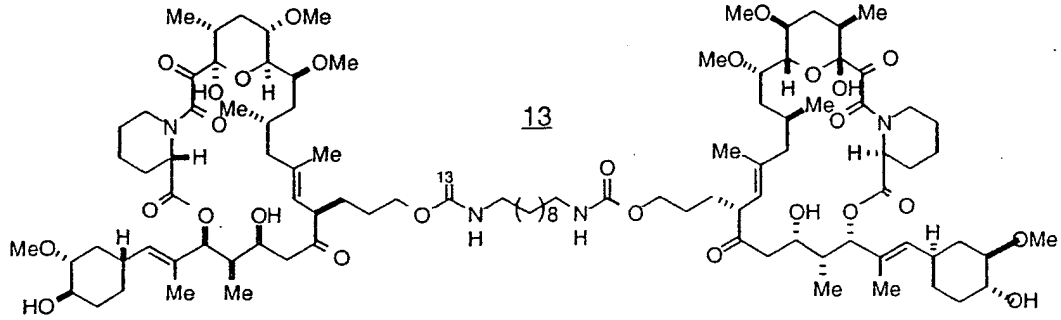

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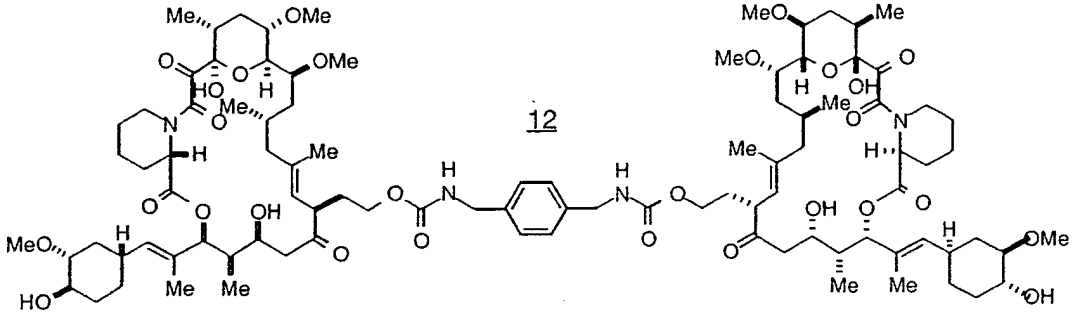

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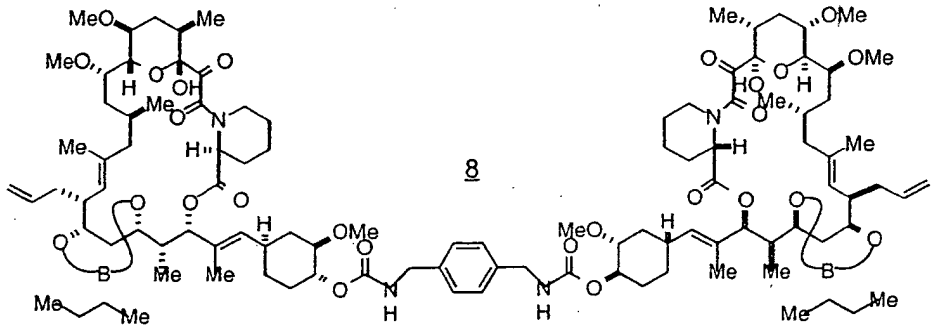

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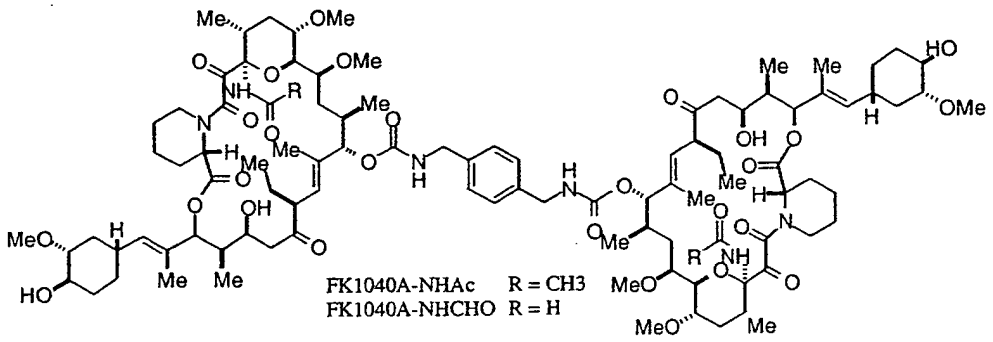

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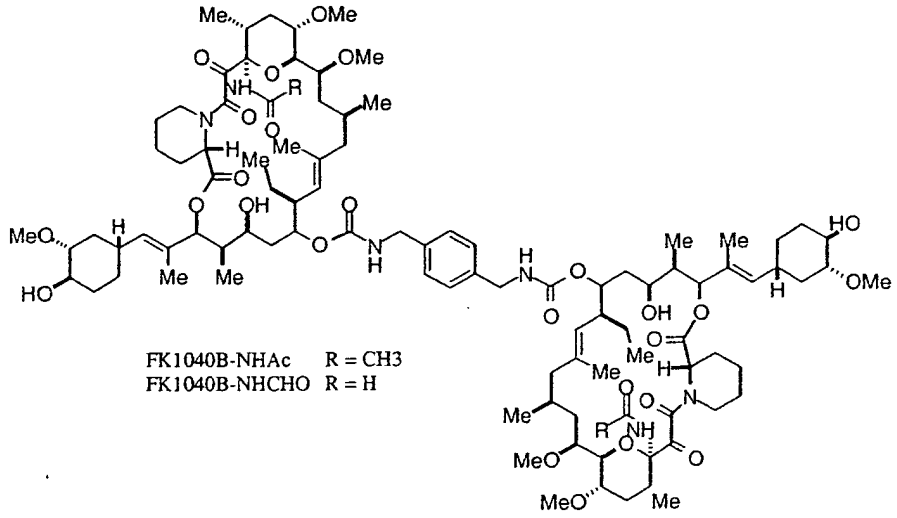

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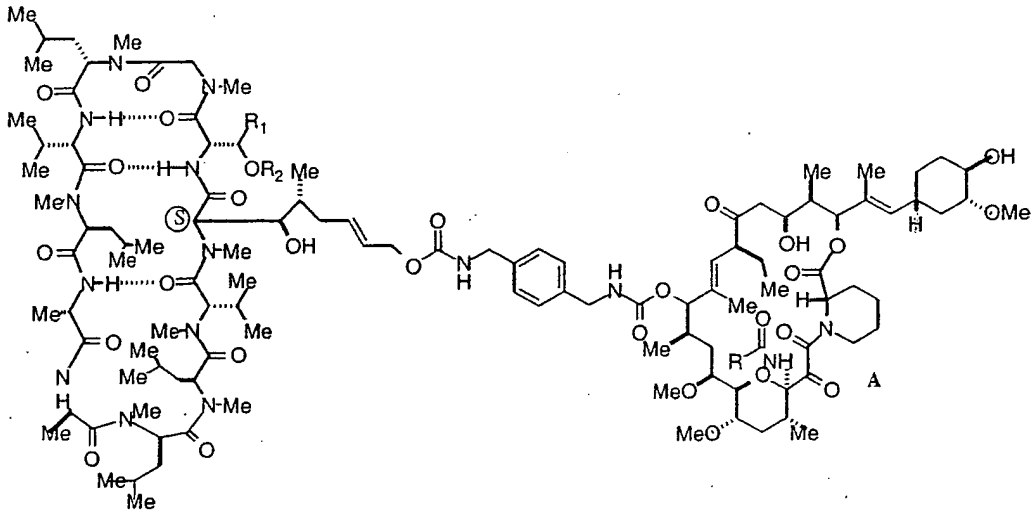

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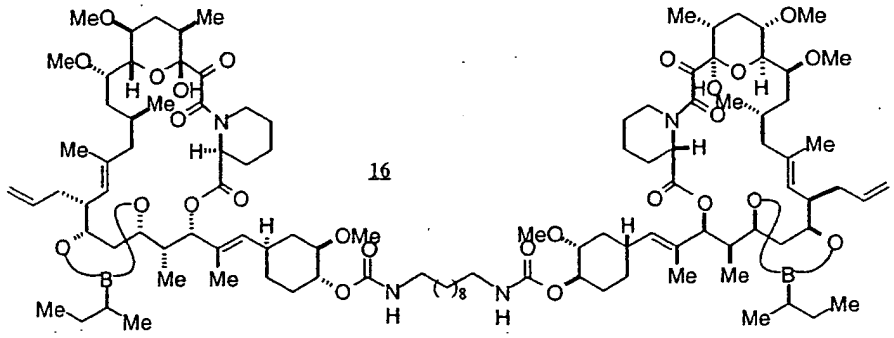

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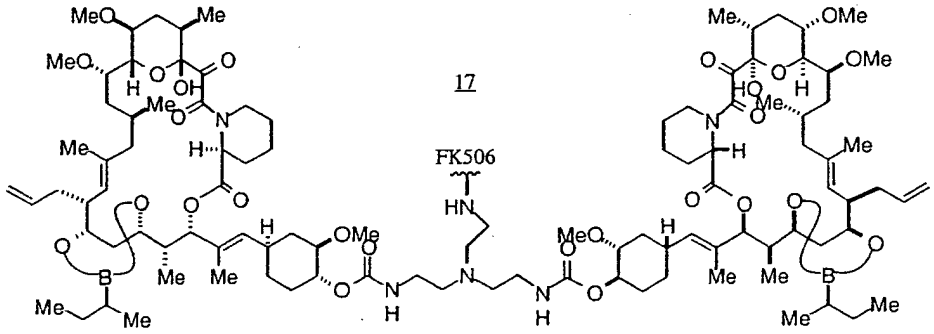

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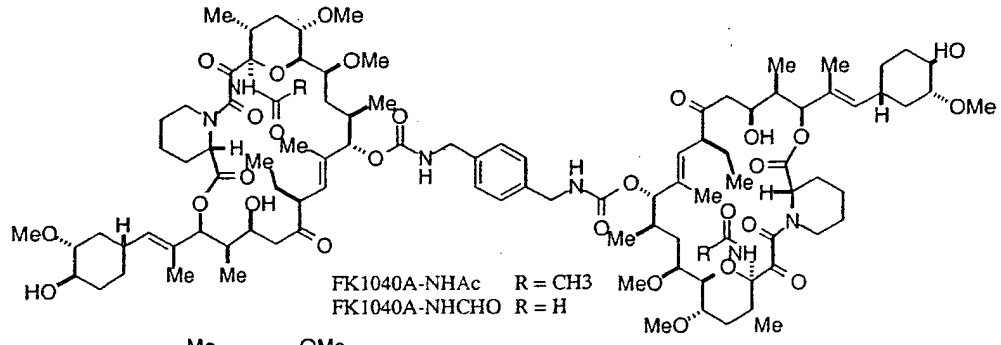

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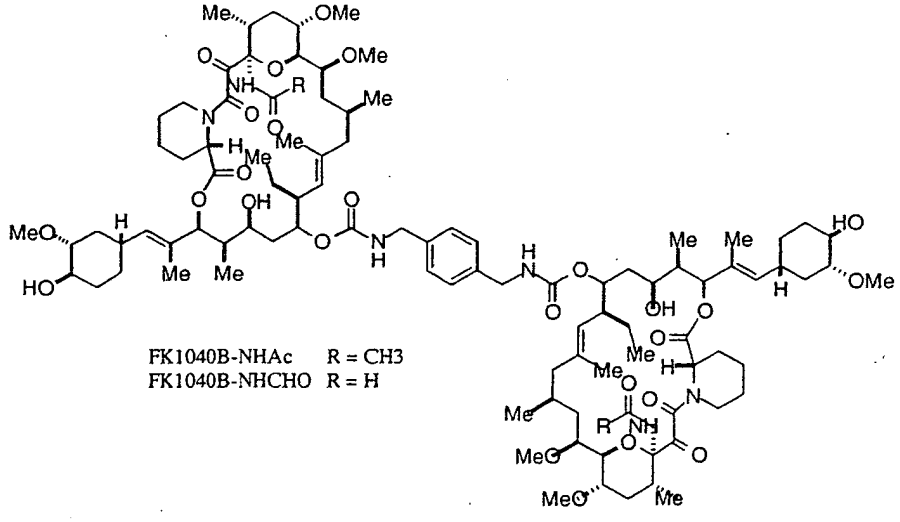

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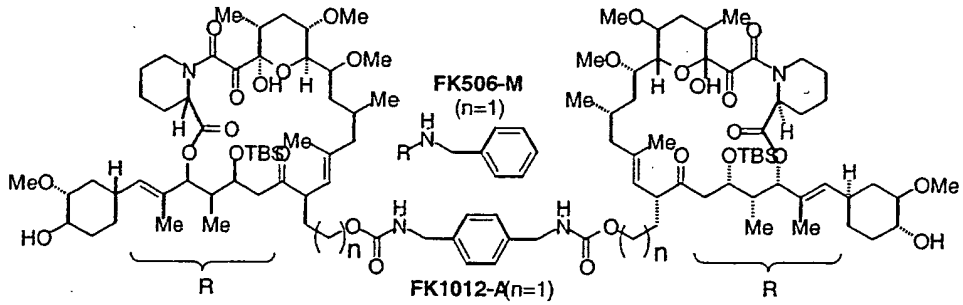
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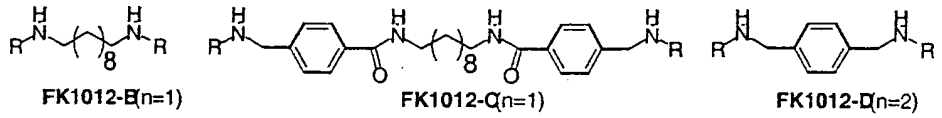
FK1012-A(n=1)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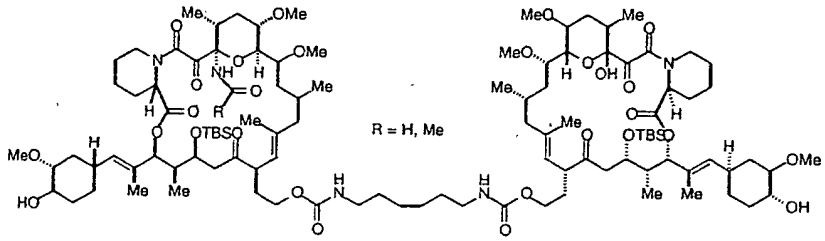
FK1012-C(n=1)
FK1012-D(n=2)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